Monitoring tumor-derived cell-free DNA in patients with solid tumors: Clinical perspectives and research opportunities

Monitoring tumor-derived cell-free DNA in patients with solid tumors: Clinical perspectives and research opportunities

Angela Esposito[a], Alberto Bardelli[b, c, d], Carmen Criscitiello[a], Nicoletta Colombo[e], Lucia Gelao[a], Luca Fumagalli[a], Ida Minchella[a], Marzia Locatelli[a], Aron Goldhirsch[f], Giuseppe Curigliano[a],*

a Division of Early Drug Development for Innovative Therapies, Istituto Europeo di Oncologia, Via Ripamonti 435, 20133 Milano, Italy
b Department of Oncology, University of Torino, Candiolo, Torino, Italy
c IRCC Institute for Cancer Research and Treatment, Candiolo, Torino, Italy
d FIRC Institute of Molecular Oncology (IFOM), Milano, Italy
e Division of Gynecologic Oncology, Istituto Europeo di Oncologia, Via Ripamonti 435, 20133 Milano, Italy
f Breast Cancer Program Istituto Europeo di Oncologia, Via Ripamonti 435, 20133 Milano, Italy

Abstract

Circulating cell-free DNA represents a non-invasive biomarker, as it can be isolated from human plasma, serum and other body fluids. Circulating tumor DNA shed from primary and metastatic cancers may allow the non-invasive analysis of the evolution of tumor genomes during treatment and disease progression through 'liquid biopsies'. The serial monitoring of tumor genotypes, which are instable and prone to changes under selection pressure, is becoming increasingly possible. The "liquid biopsy" provide novel biological insights into the process of metastasis and may elucidate signaling pathways involved in cell invasiveness and metastatic competence.

This review will focus on the clinical utility of circulating cell free DNA in main solid tumors, including genetic and epigenetic alterations that can be detected.

Keywords

- Cell free DNA;
- Plasma/serum DNA;
- Circulating nucleic acids;
- Breast cancer;
- Lung cancer;
- Colorectal cancer;
- Prostate cancer;
- Ovarian cancer

Introduction

Improvement in the clinical outcome of many cancer types is likely to be achieved by giving patients a drug tailored to the genetic makeup of their tumor. The recent surge in high-throughput sequencing of cancer genomes is delivering more accurate tumor genome information, and it can be anticipated that more

predictive biomarkers will be identified and that patients will increasingly be treated by focusing on the genetic architecture of their particular tumor rather than on the tumor's location or histological features. Circulating cell-free DNA (cf-DNA) represents a non-invasive biomarker, as it can be isolated from human plasma, serum and other body fluids [1]. Cf-DNA offer a unique opportunity for serially monitoring tumor genomes in a non-invasive manner. As cf-DNAs is a potential surrogate for the tumor itself, it is often referred to as 'liquid biopsy'.

In 1940 Mandel and Métais observed, for the first time, the presence of cell-free nucleic acid in the blood of healthy individuals [2].

Thirty years later, Leon et al. reported elevated levels of cf-DNA in the circulation of cancer patients in comparison with healthy individuals [3] and the data were confirmed in several studies [4], [5] and [6]. Subsequently, several studies reported a variety of alterations in cf-DNA such as mutations in oncogenes and tumor suppressor genes [7], microsatellite variances [8], and promoter hypermethylation [9].

The levels of cf-DNA might also reflect physiological and pathological processes that are not tumor-specific. Increased levels may be found in patients with benign lesions, inflammatory diseases and tissue trauma [10], which makes it difficult to evaluate the extent to which cf-DNA in the circulation of a patient is cancer specific.

Although the data on cf-DNA are extensive, the possible usefulness of this marker in the clinical setting remains controversial and, presently, the evaluation of cf-DNA is not yet approved in any clinical guideline.

In our review we will focus on the findings on cf-DNA in breast, lung, colorectal, ovarian and prostate cancers and discuss its relevance and potential use as diagnostic and prognostic marker.

Biology of circulating cell free DNA

The source of cf-DNA is still uncertain. A proportion seems to derive from nucleated blood cells; wild-type DNA has been detected in the plasma of cancer patients as well as in that of healthy controls. In cancer patients a considerable proportion of plasma DNA originates from tumor cells [11]. The apoptosis and necrosis of cancer cells in the tumor microenvironment are the possible explanations for the release of the nucleic acids into the blood.

Macrophages usually engulf necrotic and apoptotic cells and then they release the digested DNA into the tissue environment [11]. Another hypothesis is that cf-DNA is due to the lyses of circulating cancer cells or micro metastases shed by tumor [11]. It was also hypothesized that the tumor actively releases DNA into the bloodstream [11].

The amount of cf-DNA that derives from tumor cells changes owing to the size and the state of the tumor. The proportion of cf-DNA is also conditioned by clearance, degradation and other physiological filtering events of the blood and lymphatic circulation[12]. However the concentration of cf-DNA in serum of cancer patients is about 4 times that of healthy controls [13]. The size of the DNA released from dead cancer cells varies between small fragments of 70–200 base pairs and large fragments of about 21 kilobases and it is longer than that of non neoplastic DNA [14].

Breast cancer

Quantitative alterations of circulating DNA

Some quantitative studies reported increased concentrations of circulating DNA in patients with breast cancer when compared to healthy individuals [15], [16] and [17]. Frattini et al. demonstrated that the levels of cf-DNA present in cancer patients constitute a stable parameter over the time and its variations may be correlated with clinical outcome [15]. Large individual variations in DNA quantity have been observed among different studies. A comprehensive review reported that the concentration of DNA in the bloodstream of patients with breast cancer (BC) varied from 153 to 549 ng/ml in serum and from 122 to 462 ng/ml in plasma, whereas that of healthy controls ranged between 63–318 and 3–63 ng/ml, respectively [10].

A study by Kohler et al. showed significantly higher levels of cf-DNA in patients with BC in comparison to the patients with benign breast tumors and healthy individuals. This quantitative approach could distinguish between cancer patients and healthy controls with a sensitivity and specificity of 81% and 69%, respectively [16].

Catarino et al. quantified circulating DNA using real-time PCR (polymerase chain reaction) and showed that the cf-DNA levels were higher before breast surgery respect to post-operative time. This observation proved that cf-DNA may be a useful tool for monitoring disease. In addition, elevated levels of cf-DNA were found to be associated with tumor size, lymph node involvement, histopathological grade, and clinical staging[17]. These results were in accordance with previous observations in which the cf-DNA of BC patients also correlated with stage, lymph node metastasis and tumor size[4] and [18].

Qualitative alterations of circulating DNA

Several studies reported that a qualitative analysis of specific molecular alterations in isolated DNA from tumoral tissue may mirror cf-DNA [19]. The presence of specific mutations helps to differentiate circulating tumor DNA (ct-DNA) from normal cf-DNA. These somatic mutations, commonly single base pair substitutions, are present only in the genomes of cancer cells or pre-cancerous cells and are never present in the DNA of normal cells of the same individual. This juxtaposition confers to ct-DNA elevated specificity as a biomarker.

Four studies, for a total of 371 patients with breast cancer, analyzed the mutations of the tumor suppressor gene *TP53* [20], [21], [22] and [23].

Great variations in incidence were described in the 4 different studies, ranging from 11% to 73% and 5% to 43% for tumors and circulating DNA, respectively. These great variations should be considered as the consequence of the different methodologies used.

Also the *PIK3CA* mutations were detected in the plasma in up to 30% of patients with advanced breast cancer [24], [25] and [26]. In the study of Board and colleagues [24], the*PIK3CA* mutations were detected in cf-DNA of 13/46 (28%) patients with metastatic breast cancer but were not detected in cf-DNA of patients with operable breast cancer. The concordance was 95% for *PIK3CA* mutations found in cf-DNA compared to those found in tumor DNA. These results clearly demonstrated that the detection of cf-DNA mutations is lower in the early stage disease compared to more advanced disease and demonstrated the feasibility of detection of *PIK3CA* mutation in cf-DNA.

A more recent study showed how the analysis of ct-DNA can be integrate in the clinical management of BC patients [27].

Dawson et al. [27] compared the radiographic imaging of tumors with the assay of ct-DNA, CA 15.3 and circulating tumor cells in 30 patients with advanced breast cancer during chemotherapy administration. Ct-DNA was identified in 29 of the 30 women (97%) in whom somatic genomic alterations of *TP53* and *PIKC3A* were detected. This assay proved to have a higher sensitivity for detecting presence of metastatic disease when compared with assays of CA 15-3 or circulating tumor cells. Furthermore, the researchers found that measurement of levels of ct-DNA corresponds both with treatment response and survival: those who survived longer had lower levels of ct-DNA compared with those who survived a shorter period of time. These initial studies involved patients with high tumor burden and it remains to be evaluated if similar sensitivity can be achieved in patients with localized disease.

Additional genetic alterations that are detectable on cf-DNA and are used as biomarkers in cancer include the integrity of non-coding genomic DNA repeat sequences such as*ALU* and *LINE1*.

The *ALU* and *LINE1* sequences are involved in various physiological events such as DNA repair, transcription, epigenetic and transposon-based activity [28]and many studies showed their potential prognostic and diagnostic utility [29] and [30]. The assays are based on the observation that common DNA repeat sequences are preferentially released by tumor cells that are undergoing non apoptotic or necrotic cell death, and these fragments can be between 200 and 400 bp in size [12]. The integrity of cf-DNA *ALU* sequences in blood has been shown to be sensitive for the assessment of the early stage of BC progression, including micrometastasis [30].

Also the microsatellite instability [MSI] and loss of heterozygosity [LOH] of cf-DNA were observed to have potential application in the management of patients with BC [6],[8] and [31].

A recent study [31] showed that the detection of DNA losses of the tumor suppressor genes *TIG1*, *PTEN*, *cyclin D2*, *RB1*, and *BRCA1* on cf-DNA was associated with a more aggressive biology of BC. In particular, the detected *cyclin D2* loss was an indicator of an unfavorable prognosis. Thus, the improved detection of cf-DNA might provide clinically relevant information on the variable biology of BC. In addition, LOH was not detected in the healthy subjects whereas it was observable in patients with carcinoma in situ [6]indicating the potential application of this analysis in the screening proceedings.

Recently it has been observed that epigenetic changes are a constant alterations in cancer cells and they have a substantial effect on early carcinogenesis and progression[32]. The most examined epigenetic modification is that of DNA methylation.

A small set of genes (*APC*, *RASSF1A*, *DAP kinase*) were found hypermethylated in the cf-DNA of patients with in situ carcinoma and with benign lesions [33], suggesting that this approach may be of particular interest in view of early molecular diagnosis. In addition it was seen that hypermethylation of *RASSF1A* and *APC* detected in serum DNA of BC patients was associated with a worse outcome [34]. It was seen, also, that the combined assessment of large amounts of free circulating total/methylated DNA and circulating tumor cells was predictive for tumor progression [35]. Other studies suggested that the assessment of abnormal methylation in the serum of BC patients may used for monitoring the efficacy of neoadjuvant [36] and adjuvant [37] and [38] treatment. Therefore, the detection of methylated cf-DNA represents a promising approach for risk assessment in cancer patients.

Lung cancer

Quantitative alterations of circulating DNA

The quantitative analysis of cf-DNA as tool for the lung cancer monitoring was investigated in many studies. Significantly higher DNA concentrations were detected in the serum/plasma of lung cancer patients compared with healthy controls or patients with benign diseases [39] and [40].
In addition higher DNA levels have been reported in the serum of patients with metastatic disease than in patients with non metastatic disease, and it was seen that the cf-DNA levels decreased in 75% of the patients after therapy [3].
Sozzi et al. reported that the cf-DNA concentration was significantly lower in plasma of patients during the follow up than before surgery and was comparable to the concentration measured in the control group, suggesting that quantification of plasma DNA might represent a new approach to monitor surgical procedures or to assess the efficacy of chemo/radiotherapy [40].
Plasma DNA concentrations do not uniformly correlate with disease stage and histological subtypes [41].
Conflicting data exist regarding to the correlation of cf-DNA with survival. Some authors reported no correlation between plasma DNA concentrations and relapse-free or overall survival [40] and [41], whereas other authors reported an association of plasma DNA with survival, lactate dehydrogenase[39] and [42], and NSE [42] for a mixed group of SCLC and NSCLC patients [42], and for NSCLC patients only [39].

Qualitative alterations of circulating DNA

Several oncogenes are involved in lung carcinogenesis, such as *Ras*, *c-myc*, tyrosine-kinase receptors (*EGFR*) and c-erbB2 (*HER2*/*neu*).
Ramirez et al. reported *K-ras* mutations in 24% of 50 resected NSCLC patients [43] and Kimura et al. detected mutations in 20% of cases [44], whereas Bearzatto et al. did not find *K-ras* mutations in serum [45].
Camps et al. demonstrated that there were no significant differences between the NSCLC patients with *K-ras* mutations in the serum and those with wild-type genotype with respect to baseline patient characteristics, response rates, progression-free survival, or overall survival [46]. Instead two other studies found an association between the presence of mutant *K-ras* in serum/plasma of NSCLC patients and survival[43] and [44]. In a more large study the plasma DNA of 180 lung patients was analyzed and it was found a significant correlation between the presence of *K-ras* mutations and poor prognosis [47].
The examination of *EGFR* mutations is essential to determine an appropriate lung cancer treatment strategy. Several clinical trials endorsed that the response rate to EGFR-TKI is approximately 70% in patients with *EGFR* activating/sensitive mutations, such as exon 19 deletions or L858R mutation [48].
Many studies have focused on detection of mutations in plasma or serum, to avoid the difficulty to obtain tumor specimens.

Kimura et al. analyzed the *EGFR* mutation status in tumor and plasma of 42 patients treated with gefitinib. *EGFR* mutations were detected in the tumor samples of eight patients and in the serum samples of seven patients highlighting that *EGFR* mutation status in serum DNA was the same as in tumor samples in almost every patient. In addition, they demonstrated a correlation between the presence of

the *EGFR* mutations in plasma DNA and the objective responses to gefitinib and as well as a trend towards increased overall survival in patients treated with gefitinib [49].

The EURTAC trial [50] reported, for patients with EGFR mutations detected in serum, an hazard ratio (HR) of 0.25 in favor of erlotinib, which was similar to results from IPASS study (HR 0.29 in favor of gefitinib) [51]. Moreover, in the EURTAC trial, the subgroup analysis of *EGFR* mutations in cf-DNA showed that the presence of *EGFR* mutations was an independent prognostic marker for progression free survival (PFS) (HR 0.43, 95% CI 0.26–0.73; $p = 0.002$). The PFS for patients with mutations detected in serum was 10.7 months (95% CI 6.8–15.5) in the erlotinib group compared with 4.2 months (3.2–6.0) in the standard chemotherapy group (HR 0.25, 95% CI 0.12–0.54; $p = 0.0002$). PFS for patients in whom mutations were not detected was 12.6 months (95% CI 8.3−not assessable) in the erlotinib group compared with 6.0 months (4.9–9.0) in the standard chemotherapy group (HR 0.29, 95% CI 0.13–0.63; $p = 0.0010$) [50].

Another study demonstrated that the plasma levels of the mutant sequences of *EGFR*correlated with the clinical response and that a decreased concentration was detected in all patients with partial or complete clinical remission, whereas persistence of mutation was observed in the patient with cancer progression [52].

These studies suggest that the detection of *EGFR* and other mutations in cf-DNA could be used to monitoring response treatment and to detect the presence of new acquired mutations in plasma or serum. In 50% of lung cancer patients, acquired resistance to gefitinb or erlotinib, develops through the emergence of EGFR T790M variants [53]. The mutation at residue 790 increases the affinity of EGFR for ATP and so out-competes binding of the inhibitors. These results have been initially obtained examining biopsies from patients who relapsed upon anti-EGFR and were later confirmed through the analyses of plasma, providing the first example that resistance to targeted therapies of solid tumors can be detected non-invasively in the blood of patients [54].

It was observed also that the MSI, the LOH and the epigenetic alterations of cf-DNA may have a potential application in the management of patients with lung cancer [40], [45],[55], [56] and [57].

Colorectal cancer

Quantitative alterations of circulating DNA

The detection of circulating DNA has strengthened its role as an additional tool for the management of patients with colorectal cancer (CRC).

A study by Frattini et al. suggested that the quantification of plasma tumor DNA might be useful for monitoring patients with colorectal cancer and, prospectively, for recognizing high-risk individuals [58]. The data of this study showed that the plasma tumor DNA levels were significantly higher in patients with CRC, they decreased progressively in the follow up period in tumor-free patients and instead they increased in patients with recurrence or metastasis [58]. These results are in line with the findings of another study which demonstrated that serum DNA levels were significantly increased in stage IV CRC patients and they fluctuated during chemotherapy [59].

A study performed in a cohort of patients with CRC undergoing resection with curative intent showed that it is possible to define a unique set of molecular probes for each patient. These probes can then be

exploited to quantify tumoral DNA after surgery. The patients were subsequently followed over the course of 2–5 years. In this study, ct-DNA was shown to be sufficiently sensitive to detect minimal residual disease after surgical resection [60].

Other studies reported higher levels of circulating DNA in CRC patients than in healthy individuals, but a correlation was not demonstrated between the levels of circulating DNA and the size and site of the tumor and the clinical course of disease [61] and [62].

Another study compared the sensitivity and specificity of cf-DNA with that of conventional serum marker carcinoembryonic antigen (CEA) and it was showed that cf-DNA, when used in combination with CEA, represents a potentially useful tool for the diagnosis of early stage CRC [63].

Qualitative alterations of circulating DNA

Although anti-EGFR therapy has established efficacy in metastatic colorectal cancer (MCRC), only 10–20% of unselected patients respond [64]. This is partly due to *KRAS*mutation, which is currently assessed in the primary tumor. Many studies assessed mutation status in circulating tumor DNA, with the aim to improve patient selection [65],[66], [67] and [68].

In the study published by Bazan et al., it was shown that the preoperative detection of mutant *KRAS* and *TP53* in the serum of CRC patients undergoing resective surgery, was predictive of disease recurrence [65]. In another study it was detected the amount of circulating tumor DNA in 18 MCRC patients who underwent surgical resection of their metastases and it was observed that the detection of circulating mutant DNA after surgery was highly predictive of disease recurrence [66]. These results were agree with those of a previous study in which the probability to have a curative surgical resection has been shown to be lower in MCRC patients with measurable *KRAS* mutation in their serum [67].

Lefebure et al. detected, in the serum of unresectable MCRC, *KRAS* mutation and*RASSF2A* methylation and they observed that the presence of circulating mutant DNA was predictive of clinical outcome [68]. In a recent study was demonstrated the clinical utility of multiplex digital PCR to screen for multiple KRAS mutations in the plasma samples of MCRC patients [69] suggesting that the “liquid biopsy” is a feasible alternative to a solid tissue biopsy for identifying specific mutations.

The principal limitations of therapies that target the extracellular domain of *EGFR* is the acquisition of secondary drug resistance. In a recent study was investigated whether the detection of *KRAS* mutant alleles, in the plasma of patients become refractory to anti-EGFR therapies, may allow the early identification of individuals at risk of develop drug resistance before radiographic documentation of disease progression [70].

It was found that *KRAS* mutant alleles were detectable in the blood of cetuximab treated patients as early as 10 months before the documentation of disease progression by radiologic assessment. Of relevance, the discovery of the mechanisms of resistance to the anti EGFR antibodies therapies in CRC was simultaneously accomplished in tissue and liquid biopsy. Another noteworthy aspect of this study was the emergence of multiple different resistance mutations in the same patient [70].

Overall these results indicate that the emergence of *KRAS* mutant clones can be detected months before radiographic progression and could allow an early initiation of combination therapies that may delay or prevent disease progression.

DNA hypermethylation in serum of colorectal cancer patients has been reported to have prognostic and predictive value [61], [71], [72] and [73] and together with the assessment of DNA integrity [74] represents another promising tool for risk assessment.

Prostate cancer

Quantitative alterations of circulating DNA

We need specific, sensitive and non invasive biomarkers for prostate cancer (PCA) diagnosis that could be used in addition to prostate-specific antigen (PSA) serial evaluations. The measurement of ct-DNA as a marker of tumor dynamics over conventional PSA or even imaging studies has been actively explored.

Two studies failed to demonstrate significant differences of ct-DNA between patients with PCA and non malignant prostate disease [75] and [76]. In the first study [75] the failure could be due to the use of a less sensitive fluorometric assay, whereas other studies used a real time PCR and detected higher DNA levels in PCA patients than in non malignant prostate disease patients [77] and [78]. Cf-DNA levels were correlated with pathological stage [78], Gleason score, surgical margin status and extraprostatic extension [79]. In addition it was showed that cf-DNA increased with diagnosis of metastasis and was predictive of survival in patients with metastatic prostate cancer [75],[79] and [80].

Qualitative alterations of circulating DNA

One of the earliest genetic alterations during prostate carcinogenesis seems to be*GSTP1* CpG island hypermethylation. A recent meta-analysis of 22 manuscripts indicated good specificity (89%) but modest sensitivity (52%) of *GSTP1* differential methylation for PCA screening [81]. The detection of hypermethylated DNA is also an adverse prognostic marker: *RASSF1A*, *RARB2*, and *GSTP1* hypermethylation were correlated with the Gleason score and the serum PSA [82]. *RARB2* and *GSTP1*methylation were also correlated with the AJCC stage [82]. *GSTP1* hypermethylation was the strongest predictor of PSA recurrence following radical prostatectomy [83] and it was correlated with the Gleason score and the extent of metastasis in patients with hormone-refractory PCA [84].
Also the analysis of the fragmentation patterns of cf-DNA provide useful information in the management of prostate cancer. It was seen that the DNA integrity was higher in PCA patients than in controls [85]. Ellinger et al. demonstrated that the DNA integrity was a predictor of PSA recurrence following radical prostatectomy and that the DNA integrity may allow to recognize PCA patients and benign prostate hyperplasia patients with a specificity of 81% and a sensitivity of 68% [77].

Ovarian cancer

Quantitative alterations of circulating DNA

Ovarian cancer is the fifth leading cause of cancer death [86] in women.
The cf-DNA has been investigated as novel biomarker for diagnosis, prediction and monitoring of therapeutic response in ovarian cancer.

Kamat et al. showed that the levels of cf-DNA were elevated in patients with invasive epithelial ovarian cancer compare to the controls emphasizing the potential role of cf-DNA as an additional non invasive technique to identify women with malignant ovarian disease [87]. These findings were not consistent with a subsequent study showing that there was no statistical difference in the levels of cf-DNA between patients with ovarian cancer and patients with benign ovarian tumors [88]. One explanation for these results is the elevation of cf-DNA by other causes, including variation in the source, in fact cf-DNA in serum is reported to be higher than in plasma.

In another study it was demonstrated also the utility of cf-DNA as surrogate biomarker of therapeutic response, using an orthotopic ovarian cancer model [89]. It was seen that cf-DNA correlated with tumor burden and the levels declined with chemotherapy.

Kamat et al. investigated also the role of pre-operative total plasma cf-DNA levels in predicting clinical outcome in patients with ovarian cancer [90].The cf-DNA levels were significantly higher in patients with invasive cancer compared with women with benign ovarian tumors and controls and high cf-DNA levels correlated with aggressive phenotypic features such as high stage and high grade. In addition it was seen that cf-DNA >22,000 GE/ml was an independent predictor of poor outcome in patients with ovarian cancer, and was superior to CA 125 in predicting mortality [90]. Also another study reported a significant difference between the presence of cf-DNA in patients with high grade serous cancer compared with other histological subtypes [91].

Wimberger et al. observed a significant relationship between residual tumor load of >1 cm after primary surgery and serum DNA levels, and both parameters were associated with a higher risk of relapse and poorer overall survival [92].

Qualitative alterations of circulating DNA

As the measurement of cf-DNA levels has some limitations as screening marker for ovarian cancer, in last years the research has focused on findings of specific alterations in cf-DNA, such as methylation or microsatellite alterations and mutation of tumor suppressor genes.

In early detection of ovarian cancer, Liggett et al. found that methylation of *RASSF1A* and*PGR-PROX* in serum may be a useful biomarker to differentiate between benign and malignant ovarian tumors with a specificity of 73.3% and a sensitivity of 80.0% [93]. Another study showed that hypermethylation of at least one of six gene promoters, including *RASSF1A*, *BRCA1*, *APC*, *DAPK* and *CDKN2A*, was detected in the serum of early-stage ovarian cancer patients with 82% sensitivity; in contrast it was not reported hypermethylation in non neoplastic tissue, peritoneal fluid, or serum from 40 control women (100% specificity) [94]. Abnormal methylation of the *HMLH1* promoter has been demonstrated to correlate with poor survival after chemotherapy [95] providing the possibility to identify those patients who relapse after standard chemotherapy and who would be suitable for novel epigenetic therapies.

In a small study, tumor specific mutated *p53* DNA levels were detected in the peritoneal fluid of 28 of 30 (93%) women with intraperitoneal ovarian cancer, including all 6 cases with non malignant cells identified on cytopathology [96]. Therefore the identification of cf-DNA in peritoneal fluid compares favorably to cytopathologic evaluation, and warrants further investigation as a diagnostic marker.

In ovarian cancer patients were investigated also the MSI and the DNA integrity with promising results [97] and [98].

Conclusion and perspectives

The detection of cf-DNA provides new opportunities for management of cancer patients adding a new useful tool for diagnosis, staging and prognosis. It offers a new type of very specific biomarker that allow to identify the mutations accumulated from each tumor and to monitor the tumor burden and the response to treatment using a minimally invasive blood analyses (Table 1).

Table 1.

Detection of cf-DNA and its alterations in patients with solid tumors.

Cf-DNA alterations	Molecular alterations				
	Breast	Lung	Colorectal	Prostate	Ovary
Mutation	TP53 (28–31,37) PIK3CA (32–34,37)	RAS (63–67) EGFR (72–77)	RAS, TP53, APC (10,84,90,92–94)	–	TP53 (127)
DNA integrity	Serum DNA integrity (40,41)	–	Serum DNA integrity (100)	Serum DNA integrity (102,115)	Serum DNA integrity (129)
Microsatellite alterations	LOH and MSI (9,11,42,43)	LOH and MSI (8, 78)	–	LOH and MSI (110,112)	LOH (128)
Methylation	RASSF1A, APC, DAPK, ESR1, BRCA1, MGMT, GSTP1, Stratifin, MDR1, HSD17B4, HIC1, NEUROD1 (48–53)	P16 (65, 79) 14-3-3sigma (80)	SEPT9, ALX4, HLTF, HPP1 (84, 95–99)	GSTP1, RASSF1A, RARB2, AR (111–114)	RASSF1A, PGR PROX, BRCA1, APC, DAPK, CDKN2A, HMLH1 (124, 126)

LOH, loss of heterozygosity; MSI, microsatellite instability.

Table options

In addition cf-DNA may have particular utility in the identification of mutations associated with acquired drug resistance in advanced cancers allowing to avoid repeat biopsies and to provide an evaluation of clonal genomic evolution associated with treatment response and resistance [70] and [99].

There are, however, several questions to be answered. One crucial factor in evaluating cf-DNA is the standardization of assays and the definition of the optimal sampling specimen (serum or plasma) to obtain data more consistent and comparative between different laboratories. Actually there are different procedures both in the pre-analytical phases (blood collection, processing, storage, baseline of patients), in DNA extraction, in quantification and analysis after the DNA extraction. These variables are critical and need to be standardized for consensus analysis and reporting. Despite these technical limitations, “liquid biopsy” may provide a unique opportunity in the field of clinical cancer research and have been already embedded in the design of several clinical trials (Table 2). Correlation of qualitative and quantitative

analysis of circulating DNA with pathological response to neoadjuvant treatment in solid tumors may anticipate potential resistance to cytotoxic and biological agents during preoperative therapy. In the era of next generation sequencing detection of specific DNA mutations predicting response or resistance to targeted agents may anticipate switch to non cross-resistant therapies. The identification of circulating DNA in patients with breast cancer receiving neoadjuvant chemotherapy may predict pathological residual disease that correlate with poor prognosis in triple negative and HER2 positive breast cancer. Another opportunity to explore the role of cf-DNA is to study the "tumor dormancy" phenomenon. Analysis of cf-DNA in patients in follow up for previous diagnosis of breast cancer has provided some hints regarding tumor dormancy. Specifically identification of cf-DNA might be employed to monitor patients with breast cancer without clinically detectable disease [100]comparing the genomic profiles of germline DNA, cf-DNA and tumor DNA as defined by single nucleotide polymorphism arrays. It is essential to highlight that plasma cf-DNA and primary tumor tissue showed some similarities regarding specific tumor copy-number variations as observed by DNA amplification at several chromosome arms [100]. The biological significance of cf-DNA in patients with no evidence of clinical disease is presently unclear; further studies are required to determine whether this approach is sufficiently sensitive as a screening tool or for identification of early relapse. More importantly, the process of identifying specific DNA mutations for each patient's cancer is a laborious process that is currently too time-intensive and costly for more widespread use. Future development will have to provide a cost effective analysis mainly identifying the genes known to be recurrently mutated in each tumor. Therefore, developing standardized methodologies for cf-DNA analyses and validation in large prospective clinical studies is mandatory to implement the 'liquid biopsy' approach in the clinical management of cancer patients.

Table 2.

Ongoing clinical trials that study cf-DNA in solid tumors with therapeutic intervention.

Clinical trial	Status	Therapeutic intervention	Setting
NCT00899548	Recruiting	Predict response after systemic therapy	MBC
NCT01198743	Recruiting	Validate prognostic value of cf-DNA	Stage II–III CRC
NCT00977457	Recruiting	Predict recurrence	Prostate cancer undergoing surgery
NCT01617915	Recruiting	Correlate cf-DNA with response to neoadjuvant CT	BC candidate to neoadjuvant CT
NCT01776684	Recruiting	Evaluation of EGFR TKI resistance mechanism	NSCLC
NCT01836640	Not yet recruiting	Evaluate cf-DNA as a surrogate for tumor biopsy to identify tumor genetic alterations	MBC

CT, chemotherapy; CRC, colorectal cancers; cf-DNA, circulating cell-free DNA; MBC, metastatic breast cancer; NSCLC, non small cell lung cancer; BC, breast cancer; EGFR TKI, epidermal growth factor receptor tyrosine kinase inhibitor.

Conflict of interest

All of the authors declare no conflicts of interest.

References

[1] Utting M, Werner W, Dahse R, et al. Microsatellite analysis of free tumor DNA in urine, serum, and plasma of patients. A minimally invasive method for the detection of bladder cancer. Clin Cancer Res 2002;8:35–40.

[2] Mandel PM. Les acides nucleiques du plasma sanguine chez l'homme. CR Acad Sci Paris 1940;142:241–53.

[3] Leon SA, Shapiro B, Sklaroff DM, et al. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977;37:646–50.

[4] Zhong XY, Ladewig A, Schmid S, et al. Elevated level of cell-free plasma DNA is associated with breast cancer. Arch Gynecol Obstet 2007;276:327–31.

[5] Sozzi G, Conte D, Leon M, et al. Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol 2003;21:3902–8.

[6] Chen X, Bonnefoi H, Diebold-Berger S, et al. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res 1999;5:2297–303.

[7] Wang JY, Hsieh JS, Chang MY, et al. Molecular detection of APC, K-RAS, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. World J Surg 2004;28:721–6.

[8] Shaw JA, Smith BM, Walsh T, et al. Microsatellite alterations plasma DNA of primary breast cancer patients. Clin Cancer Res 2000;6:1119–24.

[9] Fujiwara K, Fujimoto N, Tabata M, et al. Identification of epigenetic aberrant promoter methylation in serum DNA is useful for early detection of lung cancer. Clin Cancer Res 2005;11:1219–25.

[10] Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer-a survey. Biochim Biophys Acta 2007;1775:181–232.

[11] Stroun M, Maurice P, Vasioukhin V, et al. The origin and mechanism of circulating DNA. Ann NY Acad Sci 2000;906:161–8.

[12] Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11(6):426–37.

[13] Shapiro B, Chakrabarty M, Cohn EM, et al. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer 1983;51:2116–20.

[14] Jahr S, Hentze H, Englisch S, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 2001;61:1659–65.

[15] Frattini M, Balestra D, Verderio P, et al. Reproducibility of a semiquantitative measurement of circulating DNA in plasma from neoplastic patients. J Clin Oncol 2005;23(13):3163–4.

[16] Kohler C, Radpour R, Barekati Z, et al. Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors. Mol Cancer 2009;8:105.

[17] Catarino R, Ferreira M, Rodrigues H, et al. Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer. DNA Cell Biol 2008;27:415–21.

[18] Shao ZM, Wu J, Shen ZZ, et al. P53mutation in plasma DNA and its prognostic value in breast cancer patients. Clin Cancer Res 2001;7:2222–7.

[19] Sorenson GD, Pribish DM, Valone FH, et al. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994;3:67–71.

[20] Di GH, Liu G, Wu J, et al. Peripheral blood mutated p53 DNA and its clinical value in human breast cancer. Zhonghua Zhong Liu Za Zhi 2003;25: 137–40.

[21] Silva JM, Dominguez G, Garcia JM, et al. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res 1999;59:3251–6.

[22] Silva JM, Garcia JM, Dominguez G, et al. Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. Ann Surg Oncol 2002;9:71–6.

[23] Garcia JM, Garcia V, Silva J, et al. Extracellular tumor DNA in plasma and overall survival in breast cancer patients. Genes Chromosomes Cancer 2006;45:692–701.

[24] Board RE, Wardley AM, Dixon JM, et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Res Treat 2010;120:461–7.

[25] Angenendt P, David K, Juhl H, et al. Detection of phosphoinositide-3-kinase, catalytic, and alpha polypeptide (PIK3CA) mutations in matched tissue and plasma samples from with metastatic breast cancer. J Clin Oncol 2010;28(Suppl.):a10502 [abstract].
[26] Higgins MJ, Jelovac D, Barnathan E, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 2012;18:3462–9.
[27] Dawson SJ, Tsui DW, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368(13):1199–209.
[28] Bennett EA, Keller H, Mills RE, et al. Active Alu retrotransposons in the human genome. Genome Res 2008;18:1875–83.
[29] Sunami E, Vu AT, Nguyen SL, et al. Quantification of LINE1 in circulating DNA as a molecular biomarker of breast cancer. Ann NY Acad Sci 2008;1137:171–4.
[30] Umetani N, Giuliano AE, Hiramatsu SH, et al. Prediction of breast tumor progression by integrity of free circulating DNA in serum. J Clin Oncol 2006;24:4270–6.
[31] Schwarzenbach H, Eichelser C, Kropidlowski J, et al. Loss of heterozygosity at tumor suppressor genes detectable on fractionated circulating cell-free tumor DNA as indicator of breast cancer progression. Clin Cancer Res 2012;18(20):5719–30.
[32] Dehan P, Kustermans G, Guenin S, et al. DNA methylation and cancer diagnosis: new methods and applications. Expert Rev Mol Diagn 2009;9:651–7.
[33] Dulaimi E, Hillinck J, Ibanez de Caceres I, et al. Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res 2004;10:6189–93.
[34] Muller HM, Widschwendter A, Fiegl H, et al. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res 2003;63:7641–5.
[35] Van der Auwera I, Elst HJ, Van Laere SJ, et al. The presence of circulating total DNA and methylated genes is associated with circulating tumour cells in blood from breast cancer patients. Br J Cancer 2009;100(8):1277–86.
[36] Sharma G, Mirza S, Parshad R, et al. DNA methylation of circulating DNA: a marker for monitoring efficacy of neoadjuvant chemotherapy in breast cancer patients. Tumour Biol 2012;33(6):1837–43.
[37] Fiegl H, Jones A, Hauser-Kronberger C, et al. Methylated NEUROD1 promoter is a marker for chemosensitivity in breast cancer. Clin Cancer Res 2008;14:3494–502.
[38] Fiegl H, Millinger S, Mueller-Holzner E, et al. Circulating tumour specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. Cancer Res 2005;65:1141–5.
[39] Gautschi O, Bigosch C, Huegli B, et al. Circulating deoxyribonucleic acid as a prognostic marker in non-small cell lung cancer patients undergoing chemotherapy. J Clin Oncol 2004;22:4157–64.
[40] Sozzi G, Conte D, Mariani L, et al. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. Cancer Res 2001;61:4675–8.
[41] Beau-Faller M, Gaub MP, Schneider A, et al. Plasma DNA microsatellite panel as sensitive and tumor-specific marker in lung cancer Patients. Int J Cancer 2003;105:361–70.
[42] Fournie GJ, Courtin JP, Laval F. Plasma DNA as a marker of cancerous cell death: investigation in patients suffering from lung cancer and in nude mice bearing human tumour. Cancer Lett 1995;2:221–7.
[43] Ramirez JL, Sarries C, de Castro PL, et al. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. Cancer Lett 2003;193:207–16.
[44] Kimura T, Holland WS, Kawaguchi T, et al. Mutant DNA in plasma of lung cancer patients: potential for monitoring response to therapy. Ann N Y Acad Sci 2004;1022:55–60.
[45] Bearzatto A, Conte D, Frattini M, et al. P16(INK4A) hypermethylation detected by fluorescent methylation-specific PCR in plasmas from nonsmall cell lung cancer. Clin Cancer Res 2002;8:3782–7.
[46] Camps C, Sirera R, Bremnes R, et al. Is there a prognostic role of K-ras point mutations in the serum of patients with advanced non-small cell lung cancer? Lung Cancer 2005;50:339–46.
[47] Gautschi O, Huegli B, Ziegler A, et al. Origin and prognostic value of circulating KRAS mutations in lung cancer patients. Cancer Lett 2007;254:265–73.
[48] Rosell R, Moran T, Queralt C, et al. Spanish Lung Cancer Group. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009;361:958–67.

[49] Kimura H, Suminoe M, Kasahara K, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer 2007;97(6):778–84.
[50] Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutationpositive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13: 239–46.
[51] Goto K, Ichinose Y, Ohe Y, et al. Epidermal growth factor receptor mutation status in circulating free DNA in serum: from IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non-small cell lung cancer. J Thorac Oncol 2012;7:115–21.
[52] Young TK, Chan KC, Mok TS, et al. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res 2009;15: 2076–84.
[53] Oxnard GR, Arcila ME, Sima CS, et al. Acquired resistance to EGFR tyrosine kinase inhibitors in EGFR-mutant lung cancer: distinct natural history of patients with tumors harboring the T790M mutation. Clin Cancer Res 2011;17:1616–22.
[54] Taniguchi K, Uchida J, Nishino K, et al. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res 2011;17:7808–15.
[55] Bruhn N, Beinert T, Oehm C, et al. Detection of microsatellite alterations in the DNA isolated from tumor cells and from plasma DNA of patients with lung cancer. Ann NY Acad Sci 2000;906:72–82.
[56] Liu Y, An Q, Li L, et al. Hypermethylation of p16INK4a in Chinese lung cancer patients: biological and clinical implications. Carcinogenesis 2003;24:1897–901.
[57] Ramirez JL, Rosell R, Taron M, et al. 14-3-3sigma methylation in pretreatment serum circulating DNA of cisplatin-plus-gemcitabine-treated advanced nonsmall- cell lung cancer patients predicts survival: The Spanish Lung Cancer Group. J Clin Oncol 2005;23:9105–12.
[58] Frattini M, Gallino G, Signoroni S. Quantitative analysis of plasma DNA in colorectal cancer patients: a novel prognostic tool. Ann NY Acad Sci 2006;1075:185–90.
[59] Schwarzenbach H, Stoehlmacher J, Pantel K, et al. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Ann N Y Acad Sci 2008;1137:190–6.
[60] Diehl F, Li M, Dressman D, et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. Proc Natl Acad Sci USA 2005;102(45):16368–73. Epub 2005 Oct 28.
[61] Lecomte T, Berger A, Zinzindohoue F, et al. Detection of free-circulating tumor-associated DNA in serum of colorectal cancer patients and its association with prognosis. Int J Cancer 2002;100:542–8.
[62] Boni L, Cassinotti E, Canziani M, et al. Free circulating DNA as possible tumor marker in colorectal cancer. Surg Oncol 2007;16:29–31.
[63] Flamini E, Mercatali L, Nanni O, et al. Free DNA and carcinoembryonic antigen serum levels: an important combination for diagnosis of colorectal cancer. Clin Cancer Res 2006;12:6985–8.
[64] Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337–45.
[65] Bazan V, Bruno L, Augello C, et al. Molecular detection of TP53, KRAS and p16 promoter methylation in serum of patients with colorectal cancer and its association with prognosis. Results of a 3 years GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study. Ann Oncol 2006;17: 784–90.
[66] Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.
[67] Trevisiol C, Di Fabio F, Nascimbeni R, et al. Prognostic value of circulating KRAS2 gene mutations in colorectal cancer with distant metastases. Int J Biol Markers 2006;21:223–8.
[68] Lefebure B, Charbonnier F, Di Fiore F, et al. Prognostic value of circulating mutant DNA in unresectable metastatic colorectal cancer. Ann Surg 2010;251(2):275–80.
[69] Taly V, Pekin D, Benhaim L, et al. Multiplex picodroplet digital PCR to detect KRAS mutations in circulating DNA from the plasma of colorectal cancer patients. Clin Chem 2013
[Epub ahead of print].
[70] Misale S, Yaeger R, Hobor S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 2012;486(7404):532–6.
[71] deVos T, Tetzner R, Model F. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clin Chem 2009;55:1337–46.

[72] He Q, Chen HY, Bai EQ, et al. Development of a multiplex MethyLight assay for the detection of multigene methylation in human colorectal cancer. Cancer Genet Cytogenet 2010;202:1–10.
[73] Philipp A, Stieber P, Nagel D, et al. Prognostic role of methylated free circulating DNA in colorectal cancer. Int J Cancer 2012;131:2308–19.
[74] Umetani N, Kim J, Hiramatsu S, et al. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. Clin Chem 2006;52:1062–9.
[75] Jung K, Stephan C, Lewandowski M, et al. Increased cell-free DNA in plasma of patients with metastatic spread in prostate cancer. Cancer Lett 2004;205:173–80.
[76] Boddy JL, Gal S, Malone PR, et al. Prospective study of quantitation of plasma DNA levels in the diagnosis of malignant versus benign prostate disease. Clin Cancer Res 2005;11:1394–9.
[77] Ellinger J, Bastian PJ, Haan KI, et al. Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. Int J Cancer 2008;122:138–43.
[78] Altimari A, Grigioni AD, Benedettini E, et al. Diagnostic role of circulating free plasma DNA detection in patients with localized prostate cancer. Am J Clin Pathol 2008;129:756–62.
[79] Bastian PJ, Palapattu GS, Yegnasubramanian S, et al. Prognostic value of preoperative serum cell-free circulating DNA in men with prostate cancer undergoing radical prostatectomy. Clin Cancer Res 2007;13:5361–7.
[80] Schwarzenbach H, Alix-Panabieres C, Muller I, et al. Cell-free tumor DNA in blood plasma as a marker for circulating tumor cells in prostate cancer. Clin Cancer Res 2009;15:1032–8.
[81] Wu T, Giovannucci E, Welge J, et al. Measurement of GSTP1 promoter methylation in body fluids may complement PSA screening: a meta-analysis. Br J Cancer 2011;105:65–73.
[82] Sunami E, Shinozaki M, Higano CS, et al. Multimarker circulating DNA assay for assessing blood of prostate cancer patients. Clin Chem 2009;55:559–67.
[83] Bastian PJ, Palapattu GS, Lin X, et al. Preoperative serum DNA GSTP1 CpG island hypermethylation and the risk of early prostate specific antigen recurrence following radical prostatectomy. Clin Cancer Res 2005;11:4037–43.
[84] Reibenwein J, Pils D, Horak P, et al. Promoter hypermethylation of GSTP1, AR, and 14-3-3-sigma in serum of prostate cancer patients and its clinical relevance. Prostate 2007;67:427–32.
[85] Hanley R, Rieger-Christ KM, Canes D, et al. DNA integrity assay: a plasmabased screening tool for the detection of prostate cancer. Clin Cancer Res 2006;12:4569–74.
[86] Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
[87] Kamat AA, Sood AK, Dang D, et al. Quantification of total plasma cell-free DNA in ovarian cancer using real-time PCR. Ann N Y Acad Sci 2006;1075: 230–4.
[88] No JH, Kim K, Park KH, et al. Cell-free DNA level as a prognostic biomarker for epithelial ovarian cancer. Anticancer Res. 2012;32(8):3467–71.
[89] Kamat AA, Bischoff FZ, Dang D, et al. Circulating cell-free DNA: a novel biomarker for response to therapy in ovarian carcinoma. Cancer Biol Ther 2006;5(10):1369–74.
[90] Kamat AA, Baldwin M, Urbauer D, et al. Plasma cell-free DNA in ovarian cancer: an independent prognostic biomarker. Cancer 2010;116(8): 1918–25.
[91] Dobrzycka B, Terlikowski SJ, Kinalski M, et al. Circulating free DNA and p53 antibodies in plasma of patients with ovarian epithelial cancers. Ann Oncol 2011;22(5):1133–40.
[92] Wimberger P, Roth C, Pantel K, et al. Impact of platinum-based chemotherapy on circulating nucleic acid levels, protease activities in blood and disseminated tumor cells in bone marrow of ovarian cancer patients. Int J Cancer 2011;128(11):2572–80.
[93] Liggett TE, Melnikov A, Yi Q, et al. Distinctive DNA methylation patterns of cell-free plasma DNA in women with malignant ovarian tumors. Gynecol Oncol 2011;120:113–20.
[94] Ibanez de Caceres I, Battagli C, Esteller M. Tumor cell-specific BRCA1 and RASSF1A hypermethylation in serum, plasma, and peritoneal fluid from ovarian cancer patients. Cancer Res 2004;64:6476–81.
[95] Gifford G, Paul J, Vasey PA, et al. The acquisition of hMLH1 methylation in plasma DNA after chemotherapy predicts poor survival for ovarian cancer patients. Clin Cancer Res 2004;10:4420–6.
[96] Swisher EM, Wollan M, Mahtani SM, et al. Tumor-specific p53 sequences in blood and peritoneal fluid of women with epithelial ovarian cancer. Am J Obstet Gynecol 2005;193(3 Pt. 1):662–7.

[97] Kuhlmann JD, Schwarzenbach H, Wimberger P, et al. LOH at 6q and 10q in fractionated circulating DNA of ovarian cancer patients is predictive for tumor cell spread and overall survival. BMC Cancer 2012;12:325.
[98] Salani R, Davidson B, Fiegl M, et al. Measurement of cyclin E genomic copy number and strand length in cell-free DNA distinguish malignant versus benign effusions. Clin Cancer Res 2007;13:5805–9.
[99] Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497(7447):108–12.
[100] Shaw JA, Page K, Blighe K, et al. Genomic analysis of circulating cell-free DNA infers breast cancer dormancy. Genome Res 2012;22(2):220–31.